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Discovery of Swine as a Host for the *Reston ebolavirus*

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Discovery of Swine as a Host for the *Reston ebolavirus*

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Since the discovery of the Marburg and Ebola species of filovirus, seemingly random, sporadic fatal outbreaks of disease in humans and nonhuman primates have given impetus to identification of host tropisms and potential reservoirs. Domestic swine in the Philippines, experiencing unusually severe outbreaks of porcine reproductive and respiratory disease syndrome, have now been discovered to host *Reston ebolavirus* (REBOV). Although REBOV is the only member of *Filoviridae* that has not been associated with disease in humans, its emergence in the human food chain is of concern. REBOV isolates were found to be more divergent from each other than from the original virus isolated in 1989, indicating polyphyletic origins and that REBOV has been circulating since, and possibly before, the initial discovery of REBOV in monkeys.

Filoviruses are associated with acute fatal hemorrhagic diseases of humans and/or nonhuman primates. The family consists of two genera: *Marburgvirus*, which comprises various strains of the *Lake Victoria marburgvirus* (MARV) discovered in 1967; and the antigenically distinct genus *Ebolavirus* discovered in 1976, which comprises five species including *Sudan ebolavirus* (SEBOV), *Zaire ebolavirus* (ZEBOV), *Ivory Coast ebolavirus* [also known as Cote d'Ivoire Ebola virus (CIEBOV)], *Bundibugyo ebolavirus* (BEBOV), and *Reston ebolavirus* (REBOV) (*1*). REBOV is the only member of the family thus far not associated with disease in humans (*2*).

Since the discovery of filoviruses more than 40 years ago, ostensibly random, sporadic, and fatal outbreaks of disease in primates have evoked interest in delineation of host tropisms, potential reservoirs for disease transmission, and persistence in nature (*3*). These lines of investigation have recently identified African fruit bats as potential reservoirs for ZEBOV (*4*, *5*) and MARV (*6*, *7*). Similar links to bats have been found for emerging infections in swine and humans involving paramyxoviruses and the severe acute respiratory syndrome (SARS) coronavirus (*8*, *9*).

Until now, REBOV has only been associated with disease in nonhuman primates (*2*, *10*). The virus was originally identified in 1989 in the United States from a shipment of cynomolgus monkeys (*Macaca fascicularis*) from the Philippines. Outbreaks of disease occurred in the United States in 1990 and 1996 and in Italy in 1992, which were traced back to a single facility in the Philippines (fig. S1) (*11*, *12*). Here, we report the identification of REBOV infection in domestic swine coinfected with porcine reproductive and respiratory syndrome virus (PRRSV) that were experiencing a severe respiratory disease syndrome.

In July 2008, the Philippine Department of Agriculture requested the assistance of the U.S.

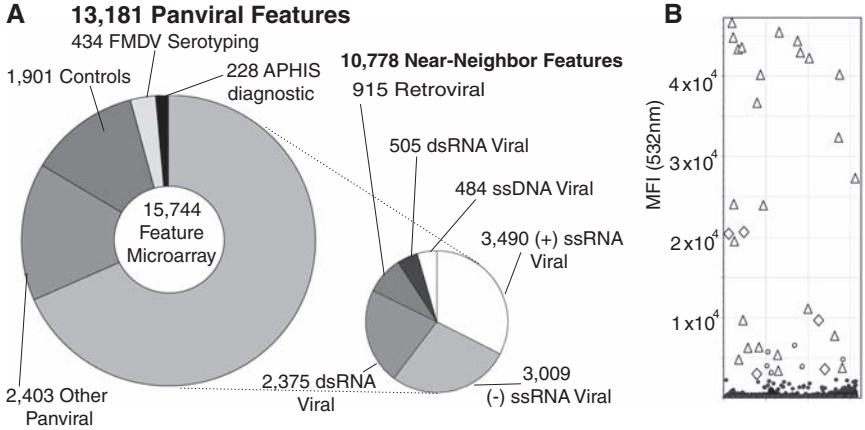

Fig. 1. Detection of REBOV in swine samples from the Philippines. (**A**) Composition of the panviral microarray used to detect REBOV. The microarray feature composition is summarized with reference to the number of unique features for identification of viral pathogens. FMDV, foot-and-mouth disease virus. (**B**) Microarray analysis of Vero cell culture of a swine lymph node from sample group A identified multiple positive features within the genus of Ebola viruses. These features corresponded primarily to sequences from REBOV with minimal reactivity toward SEBOV and ZEBOV. MFI, mean fluorescence intensity (△) Positive *Reston ebolavirus* spp. features; (◇) positive *Ebolavirus* genus features; (○) non-*Ebolavirus* features; and (●) negative features.

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Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Foreign Animal Disease Diagnostic Laboratory (FADDL), in the diagnostic investigation of recent multiple outbreaks of a respiratory and abortion disease syndrome in swine. Clinical signs resembled a highly pathogenic PRRSV infection, also referred to as "blue ear disease," which has recently been spreading through Asia (*13*–*15*). Sera and tissue samples were collected from five groups of swine at two commercial premises located in Pandi, Bulacan (sample group A); Manaoag, Pangasinan (sample groups C and E); and two inspection check points located in Sto. Nino, San Jose City, Nueva Ecija (sample group B) and Batangas (sample group D) (fig. S1). The diagnostic investigation at FADDL included diagnostics for African swine fever and classical swine fever, a directed investigation for the presence of PRRSV, and a more general search of other viral agents potentially contributing to the disease. Selected tissue samples from each group were tested and found negative for the presence of African swine fever, classical swine fever, swine vesicular disease, and foot-and-mouth disease. Consistent with a respiratory and reproductive disease syndrome, PRRSV was discovered. Sequence analysis of the *NSP2* gene revealed that it was most homologous to Chinese PRRSV isolates recently associated with blue ear disease in Asia. This determination was based on the presence of two unique deletions in the *NSP2* gene of the Philippines PRRSV isolate that are shared by recent Chinese PRRSV isolates associated with pathogenic PRRS in Asia (*14*, *16*). Simultaneously, a lymph node from group A, cultured in Vero cells, a monkey kidney cell line nonpermissive for PRRSV, revealed cytopathic effects indicating the presence of a virus other than PRRSV.

To resolve such unexplained cases, a panviral microarray has been developed that used a near-neighbor approach for the identification of taxonomically conserved viral protein microdomains. This tool consists of tens of thousands of conserved viral genetic signature sequences microscopically arrayed on a slide and is designed to capture extracted and amplified viral nucleic acid from a query sample (Fig. 1A). It is similar in concept and design to previously published pathogen microarrays, including a panmicrobial array named the GreeneChip (*17*, *18*) and the

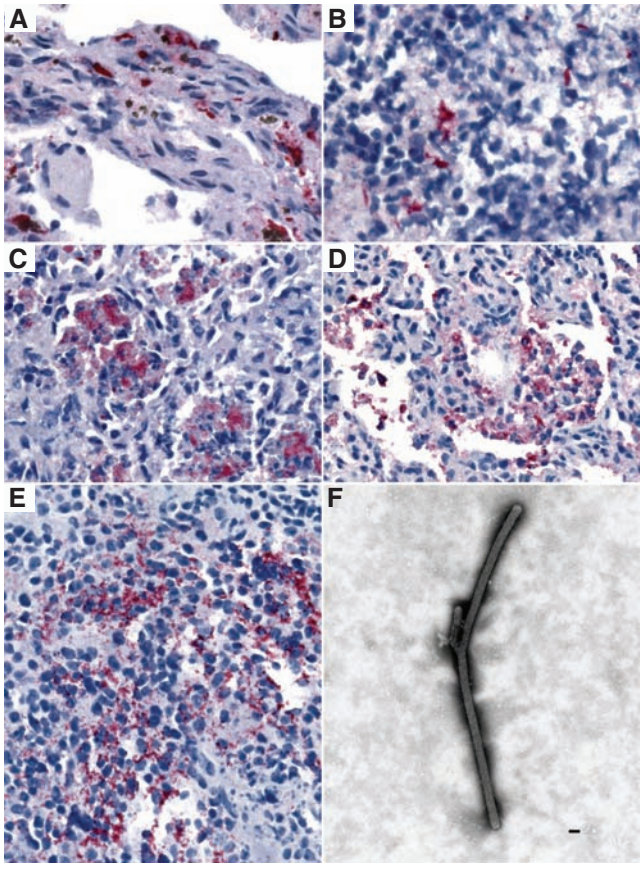

Fig. 2. Immunohistopathology of EBOV and PRRSV. (**A**) Lymph node capsule stained for EBOV antigens. (**B**) Lymph node tissue stained for EBOV antigens. (**C**) Lung tissue stained for EBOV antigens. (**D**) Lung tissue stained for PRRSV antigens. (**E**) Lymph node germinal center stained for PRRSV antigens. (A to E) Immunoalkaline phosphatase staining, naphthol fast red substrate with light hematoxylin counterstain. (**F**) Filovirus particle by negative-staining electron microscopy of the E6 Vero cell culture of the lymph node. Scale bar, 100 nm.

A

Nucleotide Similarity	Reston08-A	Reston08-C	Reston08-E	Reston96
Reston08-A				
Reston08-C	95.5%			
Reston08-E	96.9%	95.8%		
Reston96	97.7%	96.5%	98.70%	
Reston89	97.7%	96.9%	97.90%	98.80%

Amino acid changes	Reston08-A	Reston08-C	Reston08-E	Reston96
Reston08-A 5209 aa				
Reston08-C 5201 aa	193			
Reston08-E 5201 aa	122	193		
Reston96 5201 aa	98	171	64	
Reston89 5201 aa	105	148	92	61

B

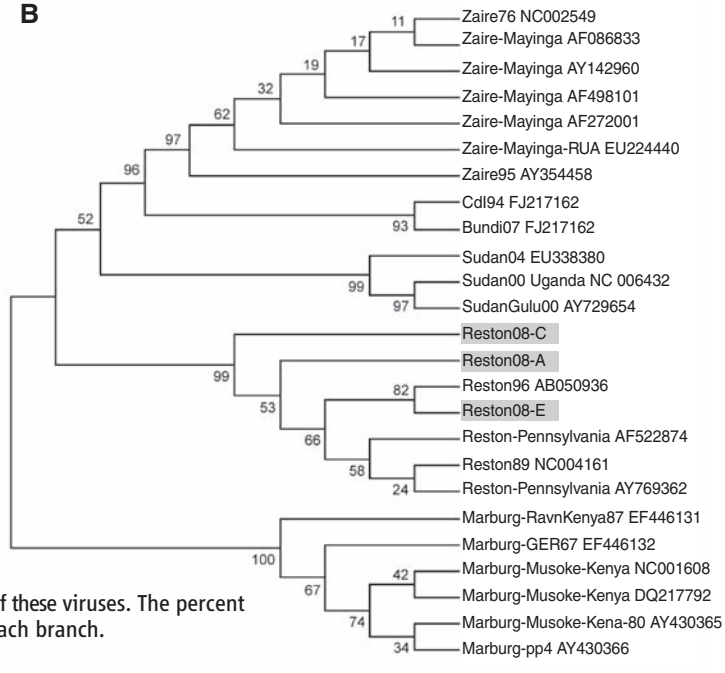

Fig. 3. Phylogeny of REBOV. (**A**) Full-length genomic sequences for Reston08-A, Reston08-C, and Reston08-E were experimentally determined, with the exception of the defined 5′ and 3′ termini, and aligned. Nucleotide similarity scores and the number of predicted amino acid changes between swine and monkey REBOV genomes are shown. (**B**) A consensus neighbor-joining tree drawn without distance topology illustrates the independent branching of the three 2008 Philippine swine viruses within the REBOV clade, demonstrating the divergence between each of these viruses. The percent branching out of 1000 random bootstrap iterations is indicated above each branch.

ViroChip used to characterize the SARS coronavirus (*19*).

To identify the unknown virus, the Vero cell culture was subjected to microarray analysis. Results revealed positive signals for 28 out of 28 distinct array features present in a 3.7-kb span of the REBOV *L* gene (Fig. 1B). By contrast, only 3 out of 30 and 2 out of 30 features were positive for the ZEBOV and SEBOV species, respectively. No other notable signals other than controls were positive (Fig. 1B). Because microarray results are sequence dependent, polymerase chain reaction (PCR) primers designed from the features themselves were used to PCR amplify and sequence the viral cDNA captured by the microarray slide. This sequence analysis confirmed that the captured viral sequences were more than 95% identical to the *L* gene of all previously sequenced REBOV isolates. Because REBOV is classified as a biological safety level 4 select agent, samples were transferred to the Special Pathogens Branch at the Centers for Disease Control and Prevention (CDC) (Atlanta, Georgia), and identification of REBOV was confirmed by Ebola-specific real-time reverse transcription (RT)–PCR analysis (table S1), antigen enzyme-linked immunosorbent assay, immunohistochemistry, and virus isolation in E6 Vero cell culture.

REBOV was only found in sample groups that also tested positive for PRRSV (table S1). Histopathological and immunohistochemical examination of lymph nodes from animals infected with REBOV and PRRSV from groups A and C showed different patterns of antigen localization and pathology (Fig. 2, A, B, and E). REBOV antigens were seen focally in lymphoid and lymph node capsule tissues with minimal necrosis (Fig. 2, A and B), whereas PRRSV antigens were seen in the germinal centers of lymphoid follicles displaying germinal cell hyperplasia and focal necrosis (Fig. 2E). Immunostaining of lung tissues for REBOV and PRRSV revealed localization of both viral antigens in areas displaying mixed inflammatory cells and sloughed necrotic debris in alveolar spaces consistent with interstitial pneumonia (Fig. 2, C and D). Negative-staining electron microscopy of the E6 Vero cell culture of the lymph node from the Bulacan site (group A) revealed filamentous virus particles and partially assembled intermediate particles characteristic of filoviruses (Fig. 2F). Serological studies on 13 swine sera from groups A, B, and D for the detection of antibodies to REBOV were negative. In contrast, antibodies to PRRSV were detected in swine sera from each of the tested sample groups A, B, and D.

RT-PCR revealed REBOV nucleic acid in animals from groups C and E, at the Pangasinan site, and from group A at the Bulacan site (fig. S1 and table S1). Samples from groups B and D did not test positive for REBOV or PRRSV; however, PCR revealed porcine circovirus type 2 (PCV-2) among samples from groups A, B, and D (table S1), and microarray analysis further revealed *Porcine teschovirus 1* from the SK6 porcine kidney cell culture of a tonsil from group D.

Viral genomes for REBOV identified from three samples—designated Reston08-A, Reston08-C, and Reston08-E—at two geographically distinct locations were ~18.9 kb in length and confirmed that the viruses were REBOV species (Fig. 3). The Reston08 viruses were significantly more divergent from each other (3.93% mean difference in nucleotide identity) than from the prototypical reference isolate from 1989 (2.5% mean difference in nucleotide identity), indicating polyphyletic origins of the REBOV infections in swine at both locations (Fig. 3A).

The lack of a phylogenetic clade, distinct from viruses in macaques, for the recent REBOV infections in swine (Fig. 3B) suggests that REBOV has been circulating since, and possibly before, the initial discovery of REBOV in monkeys exported from the Philippines in 1989. The isolation of REBOV from swine represents an extension in the known host tropism. The interisolate divergence of the three recent swine isolates is greater than that observed among the monkey isolates obtained from the single implicated primate export facility (Fig. 3A). Given the broader genetic diversity and geographic distribution of REBOV in swine, it is possible that REBOV spilled over to monkeys and swine from an as yet unidentified host. Bats have been implicated as reservoirs for other filoviruses, including ZEBOV and MARV, and may also represent a candidate reservoir for REBOV.

Of 141 tested individuals, we identified 6 individuals who worked on pig farms or with swine products that had positive serum immunoglobulin G (IgG) titers to REBOV, confirming the potential transmission from pigs to humans. The remaining 135 individuals tested negative for IgG titers to REBOV. Given the observed sequence divergence between the Reston08 viruses, a broader surveillance program is being planned. REBOV infection in domestic swine raises concern about the potential for emerging disease in humans and a wider range of livestock. However, as in previous REBOV incidents, there is no evidence of disease in humans despite the apparent occurrence of human infections evidenced by seropositive titers of REBOV-specific antibody.

The role of swine as either an incidental host or an integral part of the virus's transmission cycle has yet to be determined. Because evidence of coinfection with PRRSV, an arterivirus, was found with REBOV, we can speculate about a link between coinfection and disease in swine. This possibility is of interest in light of the atypical, highly pathogenic infections in swine by PRRSV that are currently spreading through Asia (*14*–*16*). Simian hemorrhagic fever virus, a well-known pathogen of captive primates and also an arterivirus of previously characterized pathogenicity, was identified in a coinfection of monkeys during the first detected outbreak of REBOV (*20*), although later studies clearly demonstrated the pathogenicity of REBOV as a single agent in experimentally infected monkeys (*21*).

There is concern that its passage through swine may allow REBOV to diverge and shift its potential for pathogenicity. Moreover, REBOV infections in swine highlight the need for investigations into the pathogenesis of REBOV in coinfections or in immunocompromised hosts. Through domestic and international interdisciplinary cooperation and collaboration, it is expected that future epidemiology and pathogenesis studies will shed light on the potential reservoirs, mode(s) of transmission, mechanisms of pathogenesis, prevalence of REBOV in nature, and its consequences for agricultural industries and trade.

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Supporting Online Material

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Materials and Methods
Fig. S1
Table S1
References

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This PDF file includes:

Supporting Online Material (SOM)

Materials and Methods

Panviral microarray design and analysis. A near-neighbor comparison was developed to identify taxonomically conserved viral peptide microdomains which were then used to identify 10,768 virus family, genus and/or species specific oligonucleotide features used to build the panviral microarray. (-) ssRNA virus-specific features included both sense and antisense sequences. This array was supplemented with various conserved viral genetic signatures from foreign animal disease diagnostic assays and selected viruses to achieve 13,181 unique disease pathogen features (Fig. 2A) with an estimated total theoretical detection coverage for all human, animal, avian and fish viruses with corresponding sequence in the 2007 NCBI virus nucleotide sequence database. Random primed sample cDNA was PCR amplified, labeled with biotin using a BioNick™ kit (Invitrogen) and hybridized to the microarrays at 67 ^{o}C for 16 hr in Agilent CGH microarray hybridization buffer (Agilent Technologies). Microarrays were developed with 1mM AlexaFluor–546 Streptavidin (Invitrogen) in PBST (2X PBS, 0.1% Tween-20, 1% Bovine Serum Albumin) 30min prior to a final wash and data collection using Genepix v.6.1 (BioRad).

Characterization of REBOV. Full genome sequence analyses were performed on REBOV derived from group A (lung tissue and the Vero cell culture of a lymph node, both from a single animal), group C (a pool of two lymph nodes from different animals), and group E (the Vero cell culture of a pool of three spleens from different animals). Histopathology and morphology of PRRSV and REBOV were studied by immunoalkaline phosphatase staining of formalin-fixed paraffin-embedded tissues and negative staining electron microscopy of virus from sample group A isolated from E6 Vero cell culture.

Random cDNA amplification for microarray analysis. RNA was extracted from the Vero cell culture of a lymph node from site A using RNeasy (Qiagen) and treated with DNA-free DNAse (Ambion). Random cDNA was generated using RT Superscript II (Invitrogen) and two primers GTTTCCAAGTCACGATCNNNNNNNNN and GTTTCCAAGTCACGATCTTTTTTTTTTTT. Product was treated with RNase H (Invitrogen) at 37 ^{o}C for 30 minutes followed by treatment with a QiaQuick PCR spin column (Qiagen). Random cDNA was then 3' tagged using T4 RNA ligase 1 (New England Biolabs) and a second tag primer modified by 5' phosphorylation and a 2',3'-dideoxycytidine pAAGGTTCACATTTGTAATG-ddC. Ligation was performed 4 hr at 22 ^{o}C in 1x T4 RNA Ligase 1 buffer (New England Biolabs) supplemented with 25% (w/v) PEG 8000 and 2.5 nM second tag primer (*S1*). Amplification was then performed for 36 cycles using tag complementary primers GTTTCCAAGTCACGATC and CATTACAAATGTGAACCTT. Product was cleaned using QiaQuick PCR spin columns (Qiagen).

PCR methods. Molecular detection of PCV-2 was performed from sample DNA obtained using a Qiagen DNA mini kit (Qiagen) and PCR using primers PCV-F: GCTGAACTTTTGAAAGTGAGCGGG and
PCV-R: TCACACAGTCTCAGTAGATCATCCCA with Platinum Taq Supermix (Invitrogen) and at 94 ^{o}C for 12 min followed by 40 cycles of 94 ^{o}C for 60 sec, 58 ^{o}C for 60 sec, and 72 ^{o}C for 60 sec. This yielded a 243 bp PCV specific product that was further digested with Nco-I (New England Biolabs) to distinguish PCV-2 from PCV-1.

Molecular detection of PRRSV was performed from sample RNA obtained using an RNeasy Mini kit (Qiagen) and RT-PCR using RT Superscript II followed by the Advantage 2 PCR kit (Invitrogen) using PRRSV-F: GRACTTCCTCARCTTCTTGC and PRRSV-R: TCGACGAGCTTAAAGACCAGA primers. Thermocycling conditions included denaturing at 95 ^{o}C for 2 min, followed by 35 cycles of 95 ^{o}C for 30 sec, 55 ^{o}C for 30 sec, and 68 ^{o}C for 60 sec (Guillermo Risatti, personal communication).

Molecular detection of REBOV L gene was performed from sample RNA obtained using an RNeasy Mini kit (Qiagen). RT-PCR was performed using RT Superscript II followed by Platinum Taq Supermix (Invitrogen) with primers REBOV-ER3-F: ACGAGTATATCCTGTGCACAAATCTCCTTAG and REBOV-ER3-R: CGAAGGCAGTTCCAATACTGGCAAGTGTTCC primers. PCR conditions were 94 ^{o}C for 12 min followed by 40 cycles of 94 ^{o}C for 60 sec, 58 ^{o}C for 60 sec, and 72 ^{o}C for 60 sec.

Genome amplification of REBOV was performed from extracted RNA and RT-PCR was performed as previously described (*S2*). Denaturing was 94 ^{o}C for 2 min followed by 35 cycles of 94 ^{o}C for 15 sec, primer specific annealing temperatures for 30 sec, and 68 ^{o}C for 60 sec per 1 kb of expected product size. Primers and annealing temperatures were as follows: REBOV-1F: CGGACACACAAAAAGAAA and REBOV-NP-2R-1090: CAAGAAATTAGTCCTCATCAATC, annealing at 48 ^{o}C; REBOV-NP-1F-754: GTATTTGGAAGGTCATGGATTC and REBOV-3080-R: AGTAAACACCTGCCTACAGA, annealing at 48 ^{o}C; REBOV-2841-F: ACTTACACCGGTCTATCCA and REBOV-5800-R: GAGCATTCAGAATATTGCTT, annealing at 45 ^{o}C; REBOV-5571-F: AGCAGAGGCAACAGACTC and REBOV-8640-R: GAACACGAATTTGGGATG, annealing at 47 ^{o}C; REBOV-8481-F: ATCCGGATGATGGAGCAT and REBOV-11921-R: CTGTGCACCTGTTGCCTT, annealing at 51 ^{o}C; REBOV-11741-F: TGATACACCTGTAGCAACA and REBOV-13279-R: CATAAAGTTTGGACATTCC, annealing at 43 ^{o}C; REBOV-13031-F: GGATGCAGTCTTTGAACC and REBOV-14760-R: GTACAGGTGTCTCACTGTTG, annealing at 47 ^{o}C, REBOV-14581-F: TGGAAGATGAGATGGTTTG and REBOV-18894-R: GGACACACAAAAAGGAAAA, annealing at 47 ^{o}C.

Realtime RT-PCR detection of Ebola was performed on sample RNA using Superscript III One-Step RT-PCR (Invitrogen), 50 uM forward primer AGTIACMAGTGCMTGTGGGA, 50 uM reverse primer GGCAGTTCCWATACTGGCAAGT and 10 uM probe 6-FAM-CAATCACTCAARACAGCAGCWAGIATGGC-BHQ-1 at 50 ^{o}C for 15 min and 95 ^{o}C for 2 min followed by 40 cycles of 95 ^{o}C for 15 sec and 56 ^{o}C for 45 sec. Results were recorded using an ABI7500 (Applied Biosystems).

Genomic sequence analyses. Eight overlapping RT-PCR products spanning the complete genomes of REBOV from each of the three sample groups (groups A, C and E) were generated and used for genome sequence determination. Products were treated with ExoSAP-IT® (USB Corporation), sequenced using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems), purified with CleanSeq® (Agencourt), and analyzed on the 96-capilary 3730xl DNA Analyzer (Applied Biosystems). Viral genomic ends were not experimentally determined. The Reston08-A sequence represents a composite of sequences obtained from two tissue samples from the same animal that were independently amplified and sequenced and which differ only in completeness of the 5' and 3' end regions. All shared sequence between these two independent sequence determinations (18,776 nt) were identical.

Complete genome sequences for filoviruses were aligned in CLUSTAL W using MEGA v.4.0, and 5' and 3' sequence ends were trimmed to obtain a single comparable data set. Similarity scores were calculated by Maximum Composition Likelihood and Neighbor Joining methods. DNA Distance method was applied to the tree showing branch length topology. Consensus trees were derived from 1000 bootstrap iterations.

For PRRSV, RT-PCR products corresponding to NSP2 from each positive sample group (groups A, C and E) were cloned into E. coli using TOPO-TA (Invitrogen). A partial NSP2 gene sequence representing the consensus sequence from multiple samples was submitted to Genbank under the accession number FJ641193. The putative translation of this consensus sequence was aligned with other Nsp2 PRRSV sequences in CLUSTAL W using MEGA v.4.0.

Immunohistochemical assays of REBOV and PRRSV. Immunohistochemical (IHC) assays for detection of EBOV and PRRS viral antigens were performed on formalin-fixed paraffin-embedded tissue specimens using a modification of a colorimetric indirect immunoalkaline phosphatase method (UltraVision LPValue Large Volume Detection System AP Polymer, Thermo Scientific) (*S3*) . IHC assays included a polyclonal rabbit anti-EBOV antibody, a hyperimmuned mouse anti-EBOV antibody (both from Special Pathogens Branch, CDC), and a monoclonal mouse anti-PRRS virus antibody (Rural Technologies, Inc, Brookings, SD, USA). Both anti-EBOV antibodies cross-react with all 5 species of EBOV. Appropriate positive and negative controls were run in parallel.

Serological Analyses. Analysis of sera was performed for PRRSV, CSFV, and FMDV using IDEXX HerdChek PRRS 2XR antibody ELISA kit, IDEXX HerdChek CSFV antibody ELISA kit, and Ceditest FMDV-NS ELISA (Prionics AG), respectively EBOV IgG detection was performed as previously described by the CDC.

Supporting Figure

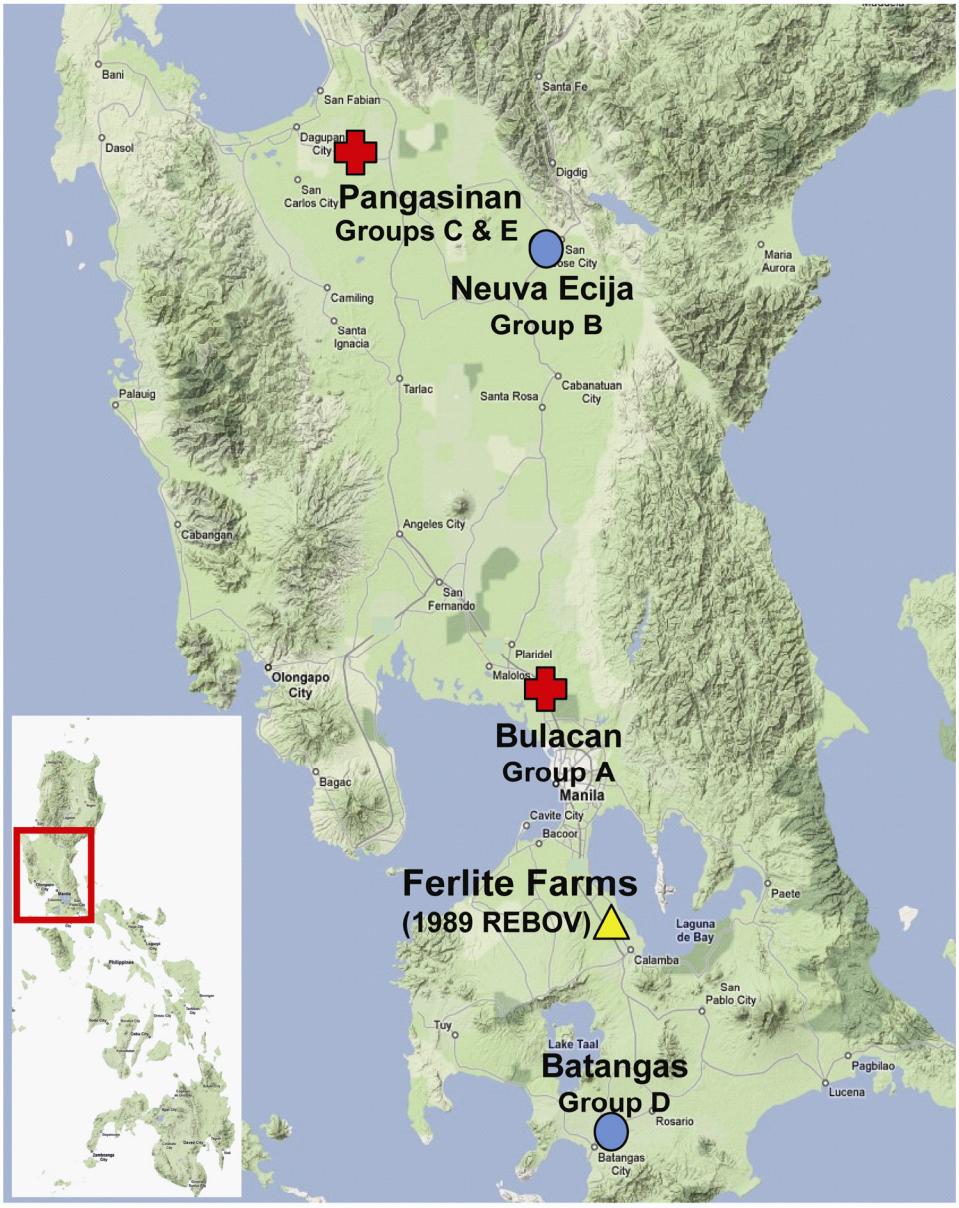

Fig. S1. A topographical map generated using Google Maps showing locations in the Philippines from where samples were obtained is shown (©2009 Google – Map data ©2009 AND Europa Technologies). Samples were collected in five animal groups designated groups A-E. The red + represent REBOV positive sites (Lat. 14°49'3.78"N; Long. 120°53'0.01"E: sample group A) and (Lat. 16° 9'35.23"N; Long. 120° 5'5.03"E: sample groups C and E) while blue O mark additional sites sampled. The site of the original isolates of REBOV in monkeys is indicated by a yellow Δ.

Supporting Table

Table S1. Swine samples and diagnostic PCRs

Group and Location	Sample Type	# Tissues /Pool	PCV-2	PRRSV	*REBOV
Group A (Bulacan)	Lung	1	+	+	+
	Spleen	1	-	-	+
	Lymph node	1	-	-	+
	Liver	1	-	-	-
Group B (Neuva Ecija)	Lung	3	+	-	-
	Spleen	3	+	-	-
	Liver	3	+	-	-
Group C* (Pangasinan)	Lung	2	-	+	+
	Lymph node	2	-	+	+
	Spleen	2	-	-	+
Group D (Batangas)	Tonsil	1	+	-	-
Group E* (Pangasinan)	Lung	2	-	+	-
	Spleen	3	-	+	+
	Tonsil	1	-	+	-

Swine samples and diagnostic PCRs. Pooled tissue homogenates of similar tissue from each of five groups were tested by PCR and found to be positive for Porcine Circovirus-type 2 (PCV-2) and Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). Additionally, conventional RT-PCR directed against the L gene of the *Reston ebolavirus* and/or Taqman realtime RT-PCR were used to test most tissue samples individually for the presence of REBOV. * For clarity, REBOV PCR results are presented as + or – based any given results for 1 or more tissues selected for the pool of tissues. Tissue pool describes the number of tissues of each type included from individual animals from their respective group. Symbols represented in the table for PCV-2, PRRS, and REBOV PCR are: + Positive or - Negative. *Groups C and E were separate groups of animals at the same premise.

Supporting References

S1. D. C. Tessier, R. Brousseau, T. Vernet, *Anal Biochem* 158, 171 (Oct, 1986).

S2. J. S. Towner, T. K. Sealy, T. G. Ksiazek, S. T. Nichol, *J Infect Dis* 196 Suppl 2, S205 (Nov 15, 2007).

S3. S. R. Zaki *et al.*, *J Infect Dis* 179 Suppl 1, S36 (Feb, 1999).